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# On the directional specificity of ribosome frameshifting at a "hungry" codon

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ABSTRACT **Limitation for aminoacyl-tRNA promotes ribosome frameshifting at certain sites. We have previously demonstrated ribosome frameshifting to the right (3′) at an AAG site in one context, and to the left (5′) at an AAG site in a different context. Here, we demonstrate that the "rightwing" context is largely specific for frameshifting to the right, and the "leftwing" context is largely specific for frameshifting to the left. Analysis of these context rules, and the conversion of a sequence that promotes leftward frameshifting to one that promotes rightward frameshifting, demonstrated here, permits us to define a minimal heptanucleotide sequence sufficient for shiftiness in each direction at an AAG codon whose lysyl-tRNA is in short supply.**

Ribosomes are normally thought to proceed in a fixed reading frame from an initiating AUG codon until termination at a stop codon. Indeed, this behavior of the translation apparatus is what gives rise to the conventional identification of an open reading frame with a gene or coding sequence. In recent years, however, many cases have come to light of ribosomes shifting their reading frame in mid-passage of a coding sequence (reviewed in refs. 1–3). Such ribosome frameshifts occur at very high frequency during translation of a class of "slippery" sequences found in retrovirus and coronavirus genomes (4–9), while slippery sequences of other kinds have been identified in the genes of certain yeast mobile elements (10, 11), in at least one bacterial mobile element (12), and in a few genes of bacteria and bacteriophages (13–20). An intriguing aspect of this widespread phenomenon is that it implies the existence of a secondary code, embedded in the conventional genetic language of translation, which provides the ribosome with instructions as to its movement along mRNA.

The *pol* gene for the protease and reverse transcriptase of retroviruses is located downstream of the *gag* gene, and in one major group its beginning overlaps the end of the *gag* gene in a different reading frame. The "hidden" reading frame for the *pol* gene in these genomes is accessed by a ribosome movement one base to the left, or 5′, direction during translation of a defined slippery sequence in the *gag–pol* overlap region (3–6, 9). A similar leftward movement is involved in the expression of the transposase gene of *Escherichia coli* mobile element IS*1* (12), the *E. coli dnaX* gene (16–18), and gene *10* of bacteriophage T7 (19, 20). In contrast, the reading frame for the reverse transcriptase of the yeast Ty element is accessed by a ribosome movement to the right or 3′ direction by one base (10, 11), and the bacterial gene encoding peptide-release factor 2 is likewise expressed by means of a rightward frameshift at an internal UGA terminator (13–15). In short, ribosomes can somehow be instructed to shift in one direction or the other, so as to access either one of the two normally unused reading frames.

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In most genes, spontaneous ribosome frameshifting is very infrequent, occurring at a frequency on the order of $10^{-4}$ (21). Leakiness of certain frameshift mutant alleles of both signs can be greatly enhanced by limitation for one or another aminoacyl-tRNA (22–24). Genetic and molecular analysis revealed that there were special sequences within which ribosomes tend to shift, in one direction or the other, at a "hungry" codon calling for an aminoacyl-tRNA in short supply (25–28). Figs. 1 and 2 present sequences within which previous work demonstrated ribosome frameshifting to the right or to the left, respectively, at an AAG codon in response to lysyl-tRNA limitation. As in the cases mentioned earlier, the assay systems initially used to identify these events generally detected only one of the alternative reading frames. In this communication we are concerned with the nature of the information which specifies the direction of ribosome frameshifting at a hungry AAG codon.

## MATERIALS AND METHODS

The host in all experiments was a derivative of *E. coli* strain CP79 (*thr leu his arg thi relA2*) with a complete deletion of the β-galactosidase gene, *lacZ*. All methods of bacterial cultivation, preparation of extracts, measurement of enzyme and protein, and purification and amino acid sequencing of β-galactosidase were done as described (26, 27).

Frameshift constructs (Table 1) were made by ligating synthetic oligonucleotides into the polylinker region of plasmid pBW1100, a derivative of pBR322 in which the tetracycline-resistance gene has been replaced by a full-length *lacZ* gene containing the pUC9 polylinker near its 5′ end (26). Complementary synthetic oligonucleotides constructed with sticky *Hin*dIII and *Bam*HI overhanging ends were ligated into the vector, which had been cut with those two enzymes.

The products of ligation were transformed into host cells as described (26, 27). Briefly, cells were grown in logarithmic phase to $OD_{700}$ of about 0.1 in Luria broth (LB), chilled, and then spun out and resuspended in 1/30th the original culture volume in LB containing 10% (wt/vol) PEG ($M_r$ 6000), 5% (vol/vol) dimethyl sulfoxide, and 20 mM $Mg^{2+}$ at a final pH of 6.5. The ligation mixture was added to 0.1 ml of these cells, held on ice for 30 min, diluted with 0.9 ml of 37°C LB plus 0.2% glucose, shaken at 37°C for 1 hr, and then plated on MacConkey agar containing carbenicillin (200 μg/ml).

Plasmids from white ($Lac^-$) colonies were analyzed by restriction with *Hin*dIII and *Pst* I. Plasmids that had lost the *Pst* I site within the pUC9 polylinker and had reformed the *Hin*dIII site were chosen. Plasmid sequences were confirmed by using a Sequenase kit and protocol from United States Biochemical.

## RESULTS AND DISCUSSION

**Directional Specificity.** Leftward frameshifting at the sequences shown in Fig. 2 *A* and *B* was detected in a leftward

Abbreviations: A site, aminoacyl site; P site, peptidyl site.

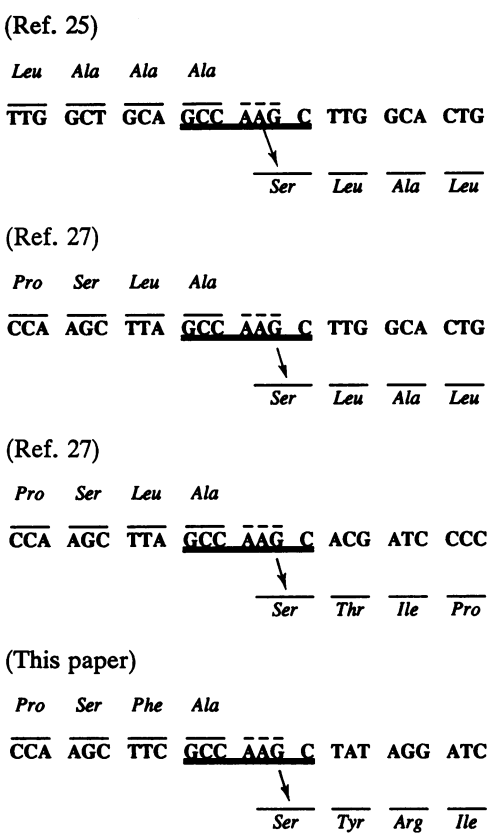


FIG. 1. Rightwingers: sequences shown to be shifty to the right during lysyl-tRNA limitation. In each case, cells were subjected to lysine-hydroxamate inhibition, shifting was detected in *lacZ* reporter constructs, and the shift position was confirmed by direct protein sequence analysis of β-galactosidase. Protein sequence data locating the frameshift site are cited for each case. The mRNA sequence in the vicinity of each frameshift site is shown in the central boldface line. The amino acids found before the shift, encoded in the normal or initiating reading frame, are quoted above the sequence line, aligned above their base triplets. The amino acids found after the shift, encoded in the shifted reading frame, are shown below the sequence line, aligned under their respective triplets. *D* presents the protein sequence around the frameshift site (positions 5–13) of β-galactosidase encoded by RW-1(right) of the present study, made in lysine hydroxamate (200 μg/ml). It was purified as described (26, 27) and sequenced at the Genetic Engineering Facility, University of Illinois.

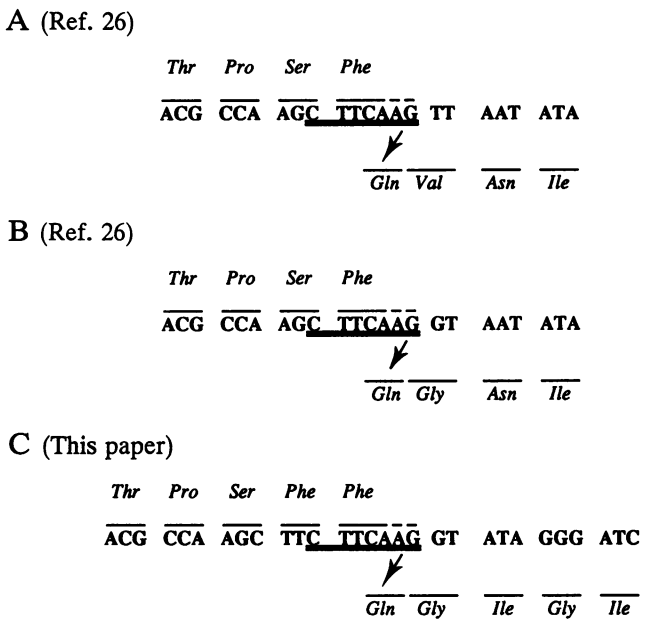


FIG. 2. Leftwingers: sequences shown to be shifty to the left during lysyl-tRNA limitation. The representation is as in Fig. 1. The construct shown in *A* was referred to as C-Oc in ref. 26; that shown in *B* was referred to as C-Am. *C* presents the protein sequence around the frameshift site (positions 4–13) of β-galactosidase encoded by LW-1(left) of the present study, made in the presence of lysine hydroxamate (100 μg/ml). It was purified as described (26, 27) and sequenced at the Genetic Engineering Facility, University of Illinois.

reporter—namely, an engineered *lacZ* gene in which active enzyme synthesis depended upon a leftward ribosome frameshift (26). The reporter gene was designed in this way so as to model phenotypic suppression of (−)-frameshift mutant alleles which had earlier been analyzed genetically (23). Fig. 3 shows the effects of lysyl-tRNA limitation on several such reporters of leftward frameshifting. The open squares, marked 2B(left), illustrate the extent of leftward frameshifting in a construct reported on previously, shown in Fig. 2*B*. The large increase in the differential rate of enzyme synthesis is to be contrasted with a 30–40% decrease observed under the same limitation in a zero-frame *lacZ*$^+$ control in the same plasmid (figure 4 of ref. 26).

The first two sequences in Fig. 2 share a CTTC quadruplet to the left of the hungry codon. These four positions, which can be expected to interact with peptidyl-tRNA when the hungry codon is in the aminoacyl (A) site, have been shown to play a large role in leftward shifting (28). To test whether any sequence further to the left matters, we made construct LW-1(left) (Fig. 2*C*). Here, the addition of a TTC after the *Hin*dIII site produces a sequence upstream of the hungry codon which retains the CTTC quadruplet on its immediate left but is different at every position further to the left (see Fig. 2 *B* and *C*). In this construct, enzyme synthesis responded to lysyl-tRNA limitation much as it did in 2B (Fig. 3, open circles), and protein sequence analysis confirmed the location of the leftward frameshift (Fig. 2*C*). Construct LW-2(left), which differs slightly from LW-1(left) downstream of the hungry codon, a point we will return to later, behaved identically (Fig. 3, open triangles).

The filled circles in Fig. 3, marked RW-1(left), refer to a construct which is identical to LW-1(left) except that the hungry codon is preceded by GCC and followed by C, a context typical of rightwingers (see Fig. 1). The (left) in the designation, as in the others, means that enzyme synthesis requires, and therefore reports on, a leftward shift by one base. Fig. 3 shows that RW-1(left) exhibited much less leftward frameshifting than LW-1(left). Evidently, the context around the hungry codon in RW-1 is not conducive to leftward frameshifting.

It is, however, highly conducive to rightward frameshifting. This is shown in Fig. 4, where each sequence tested is in a construct which requires, and therefore reports on, rightward frameshifting to yield active enzyme. Lysyl-tRNA limitation induced a large increase in enzyme synthesis from RW-1(right) (open squares), and protein sequence analysis confirmed the location of the frameshift (Fig. 1*D*). In contrast, LW-1(right) exhibited virtually no frameshifting in the rightward direction (Fig. 4, filled triangles). Thus, the responses of LW-1 and RW-1 in reporters of frameshifting in each direction (Figs. 3 and 4) show that the former context is almost completely specific for leftward frameshifting, and the latter is largely specific for rightward frameshifting. Since these two sequences differ at only three positions (Table 1), these positions (or a subset of them) must contain the information which specifies the *direction* of frameshifting at the hungry lysine codon. We have not attempted to test all possible combinations of bases at these positions for their influence on frameshift direction.

Table 1. Constructs used in this study

| Designation (shift direction reported) | Oligodeoxynucleotide (5′ to 3′) |
|---|---|
| 2B(left) | AGC-TTC-AAG-GTA-ATA-TAG-G-(GATC) |
| LW-1(left) | AGC-TTC-TTC-AAG-GTA-TAG-G-(GATC) |
| LW-2(left) | AGC-TTC-TTC-AAG-CAT-TAG-G-(GATC) |
| RW-1(left) | AGC-TTC-GCC-AAG-CTA-TAG-G-(GATC) |
| LW-1(right) | AGC-TTC-TTC-AAG-GTA-TAG-(GATC) |
| RW-1(right) | AGC-TTC-GCC-AAG-CTA-TAG-(GATC) |
| RW-1(ACC)(right) | AGC-TTC-ACC-AAG-CTA-TAG-(GATC) |
| RW-1(GAC)(right) | AGC-TTC-GAC-AAG-CTA-TAG-(GATC) |

For each construct, a pair of synthetic oligonucleotides was force-cloned into vector pBW1100 as described (26, 27). The member of each pair of oligodeoxynucleotides carrying the translated strand is shown above, with the *Hin*dIII site at left (5′); the *Bam*HI site at right (3′) in parentheses was in the vector, with its complement in the complementary oligonucleotide which is not shown. The AAG codon which was starved during growth in lysine hydroxamate is underlined. In each case, there is a TAG terminator triplet blocking the outgoing reading frame two codons after the AAG, or three codons after it in the first construct listed. This construct is designated 2B(left) here, referring to its amino acid sequence quoted in Fig. 2*B*, and was described fully in ref. 26. We list it here for comparison with the new constructs, which are all the others in this table.

It will be observed in Figs. 3 and 4 that there is a small component of starvation-induced frameshifting which is not directionally specific. That is, we see a small increase in enzyme synthesis with a rightwinger context in a leftward reporter [RW-1(left) in Fig. 3] or a leftwinger context in a rightward reporter [LW-1(right) in Fig. 4]. We have not located the frameshift event(s) responsible for this background effect by protein sequencing, and multiple events may be involved. The discussion in this paper focuses on the much larger starvation-induced frameshifting effect which is directionally specific.

**Context Determinants for Rightward Shiftiness.** Our results confirm that a GCC to the left (5′) and a C to the right (3′) of a hungry AAG codon comprise sequence information sufficient to make it shifty to the right, in agreement with the conclusions we drew from earlier constructions (27). In ref. 27, we showed that rightward shiftiness was insensitive to various changes at all positions other than these. In the present experiments, we have changed the leftwinger sequence LW-1 at *only* these positions to produce RW-1, which is sufficient to make the sequence a rightwinger (Figs. 3 and 4). These positions and the hungry codon are doubly underlined in Fig. 1. It can be seen that these are the only positions common to all four rightwingers. However, the rightward specificity of the sequence GCC-(hungry codon)-C established here does not exclude the possibility that other contexts with rightist inclinations may exist: we have not attempted to test all 256 of the possible combinations of bases at the four critical positions.

We have shown earlier that the C to the right is necessary for its shiftiness: replacement of this base by A or G abolished shiftiness, and replacement by T reduced it markedly (25). The significance of this base in the mechanism of rightward shifting is no doubt that it becomes the wobble base of the AGC triplet decoded in the rightward reading frame in place of the hungry AAG in the zero frame. The tRNA which decodes AGC has a curious weakness for frameshift errors at several different sequences (27, 29, 30).

Each position of the GCC triplet adjoining the hungry codon on the left is also necessary for high-level shiftiness.

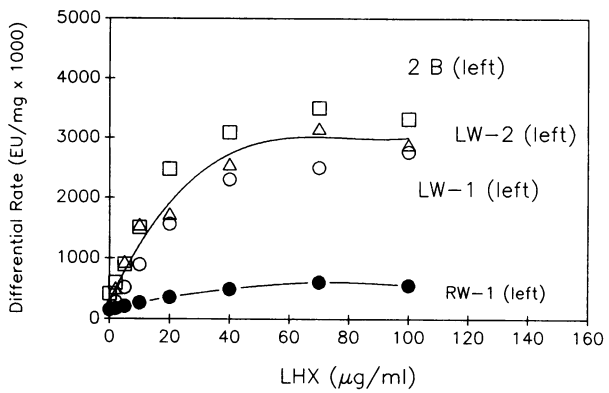


FIG. 3. Assay of leftward frameshifting. The constructs are all in leftward reporters, in which β-galactosidase synthesis depends on a one-base frameshift to the left before the blocking UAG terminator triplet two codons after the AAG lysine codon. Sequences and methods of construction are shown in Table 1. Exponential cultures were subjected to various concentrations of lysine hydroxamate (LHX), an inhibitor of lysyl-tRNA synthetase (25–27). Each point is the differential rate of β-galactosidase synthesis during about one mass doubling, averaged from two or more experiments. EU, enzyme units. Methods of cultivation and of β-galactosidase and protein assay were as described (26, 27). 2B(left) refers to the construct from an earlier study (26) described and referenced in Fig. 2*B*, which presents its protein sequence in the region of the frameshift. The data for this construct (□) were averaged from two older experiments, reported in ref. 26, and one recent repeat. LW-1(left) (○) is described in Table 1; its protein sequence is given in Fig. 2*C*. LW-2(left) (△) is identical to LW-1(left) except for the three bases immediately following the hungry codon (see Table 1). RW-1(left) (●) is identical to LW-1(left) except that the hungry codon is preceded by GCC and followed by C (see Table 1).

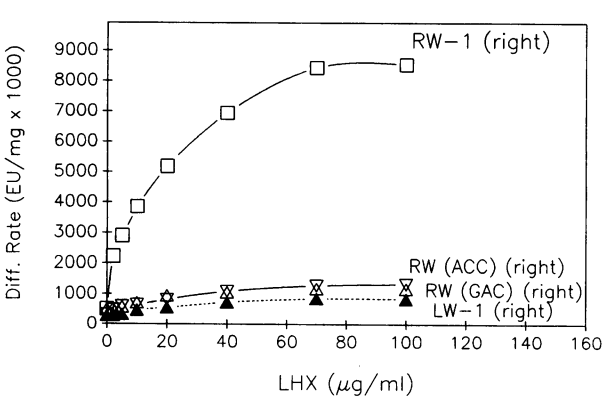


FIG. 4. Assay of rightward frameshifting. The constructs are all in rightward reporters, in which β-galactosidase synthesis depends on a one-base frameshift to the right before the blocking UAG terminator triplet. Data and methods are as in Fig. 3, and sequences are shown in Table 1. RW-1(ACC)(right) (▽) is identical to RW-1(right) (□) except that the G three positions 5′ of the hungry codon is replaced by A (see Table 1). RW-1(GAC)(right) (△) is identical to RW-1(right) except that the C two positions 5′ of the hungry codon is replaced by A (see Table 1).

Replacement of the last base of this triplet greatly diminishes shiftiness (27). In the present series of experiments, we have tested replacement of the first and second positions of this triplet. Fig. 4 shows the behavior of constructs which are identical to RW-1(right) except that the first base was changed to A in RW-1(ACC)(right) and the second base was changed to A in RW-1(GAC)(right). Both of these constructs show very little rightward shiftiness.

The mechanism by which the GCC triplet to the left of the hungry codon has its effect is unknown. This triplet is in the ribosome peptidyl (P) site when the frameshift occurs at the hungry codon, and so it is likely that the tRNA it encodes, $tRNA^{Ala}_{GCY}$, in peptidyl-tRNA form, is involved. It is not clear how. Rightward frameshifting during translation of the yeast Ty*1* transposon depends upon a critical seven-nucleotide sequence disposed in the same way with respect to the frameshift site as our rightwing critical sequence (10, 11). The base sequence of the Ty*1* heptanucleotide suggests that rightward slippage of the peptidyl-tRNA in the P site is involved in the rightward slip at the A site (11). However, the sequence of our critical site makes this mechanism appear unlikely in the present case.

If the peptidyl-tRNA in the P site slipped rightwards itself, its anticodon would be associated with the triplet CCA. This would entail mismatches in the first and third positions between the message sequence and the CGG anticodon of $tRNA^{Ala}_{GCY}$. We doubt that normal base-pairing only in the middle position of the codon/anticodon couple could account for the strong frameshifting we observe.

Moreover, the same base-pairing in the middle position only would be available to a right-shifted peptidyl-$tRNA^{Thr}_{ACY}$, the species in the P site in our construct RW-1(ACC)(right). Yet this construct exhibits much less frameshifting than RW-1(right). This consideration and the strong influence of the third base of the P-site triplet (27) both suggest that some feature of $tRNA^{Ala}_{GCY}$ other than its anticodon is involved. We have speculated elsewhere (27) on what this feature might be.

The only general conclusion we would emphasize here is that the triplet in the P site is crucially involved, somehow, in rightward ribosome frameshifting at the adjacent A site.

**Context Determinants for Leftward Shiftiness.** The leftwinger sequences we have characterized previously share a CTTC quadruplet just to the left of the hungry codon (see Fig. 2). Our new construct, LW-1(left), was designed to retain this feature while changing the entire sequence further to the left or 5′ side. This construct exhibits strong leftward frameshifting (Fig. 3), and the location of the frameshift at the lysine codon was confirmed by protein sequence analysis (see Fig. 2*C*). Thus, leftward frameshifting is independent of sequence more than four bases 5′ of the hungry codon.

Construct LW-2(left) was designed to test the relevance of context on the other (3′) side of the hungry codon. It differs from LW-1(left) at each of the three positions following the hungry codon on this side and shows an identical response to lysyl-tRNA limitation. The limited number of constructs we have tested disclose no sign of sequence specificity for leftward frameshifting in the entire region 3′ of the hungry codon. The base adjoining the hungry codon on the 3′ side is G in sequence 2B and LW-1, it is C in LW-2, and it is T in a construct studied earlier (Fig. 2*A*), all of which exhibit strong leftward frameshifting. Two alternative bases can be found in one or another of these four leftwingers at each position from the 2nd to the 14th base beyond the hungry codon. It thus appears that no single sequence on the 3′ side plays a specific role in leftward frameshifting, although we have not attempted exhaustive testing of every position.

The CTTC quadruplet immediately to the left of the hungry codon is critically involved in the ribosomal frameshift. The last base of the quadruplet, which is the position immediately neighboring the hungry codon on the left side, becomes the first base read in the shifted reading frame; we have shown that replacement of this base strongly affects shiftiness (26). A mutational analysis of the quadruplet's role in leftward frameshifting (28) strongly suggests that a P-site shift is associated with the A-site shift at the adjacent hungry codon. In brief, leftward frameshifting at the hungry A site is facilitated by sequences in the quadruplet which permit leftward slippage of the peptidyl-tRNA in the P site, and is reduced by sequences which prevent it (28). These sequence requirements in the P site resemble those which determine the natural shiftiness of certain retroviral frameshift sites (3–6). In the latter case, "simultaneous slippage" (5) of the tRNAs in the P and A sites is evidently the mechanism of frameshifting, whereas in the present case limitation for the tRNA demanded in the A site permits a different tRNA to decode the leftward overlapping triplet. Whether this is a fundamental difference or not is not clear.

**General Conclusions.** In Figs. 1 and 2 we have summarized the aforementioned minimum sequence requirements by double underlining. In each case, the critical sequence comprises seven nucleotides, including the hungry codon itself, disposed differently depending on the direction of the shift. For rightwingers, the critical heptanucleotide includes three bases to the left of the hungry codon and one to its right. For leftwingers, the critical heptanucleotide includes four bases to the left and none to the right.

The hungry codon itself dictates stalling of the ribosome for lack of cognate aminoacyl-tRNA. The system's interest lies in its clear definition of the position of ribosome stalling in relation to the other sequence elements—apparently just a few adjacent bases—which dictate the ribosome's tendency to slip left or right to decode a triplet overlapping the hungry codon from one side or the other.

The basic phenomenon of ribosome frameshifting at a hungry codon, first described in outline a decade ago (22), may therefore provide a model for the ribosome dynamics that underly programmed frameshifting at naturally shifty sequences such as those of animal virus genomes. In most (4, 6–9) but not all (31) of these latter cases, baroque secondary structures downstream of the shift sites contribute to their leftward shiftiness. The mechanism of this effect is unclear, but one plausible conjecture is that the regions of secondary structure stall a queue of ribosomes, which in turn promotes frameshifting within the queue (6). None of the constructs we have studied contain extensive regions of secondary structure (long stems or pseudoknots) downstream of the frameshift site. However, our frameshift sites are at hungry codons, and the short sequences around them which predispose them to shiftiness under the conditions we examine are thus necessarily specific for the behavior of stalled ribosomes.

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